

REMARKS

Claims 1-69 were pending in the application. Claims 15-69 have been cancelled, without prejudice, as being directed to a non-elected invention and claims 1-14 have been amended and new claims 70 and 71 have been added. Claims 2-3 and 9-10 have been withdrawn from consideration as being directed to a non-elected species. Accordingly, upon entry of the present amendment, claims 1-14 and 70-71 will remain pending in the application.

Support for the amendments to the claims may be found throughout the specification and the claims as originally filed. In particular, support for the amendments to claims 1-4 and 8-11 can be found, at least at, page 66, line 4 through page 70, line 7 of the specification and in Tables 2A and 2B. Further support for the amendments to claims 4 and 11 may be found, at least at, page 14, line 8 of the specification. Support for the amendments to claims 5-6 and 12-13 can be found, at least in, Tables 1-2B. Support for the amendments to claims 7 and 14 can be found, at least at, page 7, lines 26-29 of the specification. Support for new claims 70 and 71 can be found, at least at, page 7, lines 30-31 of the specification and in Tables 2A and 2B. No new matter has been added by the foregoing amendments to the specification and claims.

Any amendments to and/or cancellation of the claims were done solely for the purpose of expediting prosecution of the present application. Applicants reserve the right to pursue the subject matter of the claims as originally filed in this or a separate application(s). No new matter has been added to the application.

Objections to the Oath

The Examiner has objected to the oath/declaration as being defective for not identifying the present application by application number and filing date.

As evidenced by a copy of the oath/declaration that was filed on November 18, 2002 (submitted herewith), the oath does identify the present application by application number and filing date. Accordingly, Applicants respectfully request reconsideration and withdrawal of this objection to the oath.

Objections to the Specification

The Examiner has objected to the specification because, according to the Examiner, there are hyperlinks and improperly demarcated trademarks in the specification.

Responsive to the Examiner's objection, Applicants submit herewith a substitute specification in which such hyperlinks have been removed and the improperly identified trademarks have been corrected. Accordingly, Applicants respectfully request reconsideration and withdrawal of this objection to the specification.

Objection to the Claims

The Examiner has objected to claims 1, 4-8, and 11-14 as being drawn to the subject matter of non-elected species of the invention.

Applicants hereby acknowledge the election of the species of SEQ ID NO:16 and the species of combination of agents consisting of a taxane compound and a platinum compound. It is Applicants' understanding that upon the allowance of a generic claim, Applicants will be entitled to consideration of claims to additional species that are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 C.F.R. §1.141 *et seq.* Claims 1, 4-8, 11-14, and 70-71 read on the elected species of a combination of agents consisting of a taxane compound and a platinum compound. Claims 1, 5, 6, 8, 12, 13, 70, and 71 read on the elected species of SEQ ID NO:16.

Rejection of Claims 5, 6, 12, and 13 Under 35 U.S.C. §112, Second Paragraph

The Examiner has rejected claims 5, 6, 12, and 13 under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. In particular, the Examiner is of the opinion that claims 5, 6, 12, and 13 are indefinite for not reciting a step for determining the level of expression of the marker.

Applicants respectfully traverse the foregoing rejection on the grounds that the ordinarily skilled artisan would find claims 5, 6, 12, and 13 to be clear and definite based on the plain language of the claims, the teachings in Applicants' specification, and the general knowledge in the art. However, in the interest of expediting examination and allowance of the pending claims, claims 5, 6, 12, and 13 have been amended, thereby rendering the foregoing rejection moot. Accordingly, Applicants respectfully request that the aforementioned rejection be reconsidered and withdrawn.

Rejection of Claims 1, 4-8, and 11-14 Under 35 U.S.C. §112, First Paragraph

The Examiner has rejected claims 1, 4-8, and 11-14 under 35 U.S.C. §112, first paragraph as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. In particular, the Examiner objects to the claims on the grounds that "...ovarian cancer cells are not representative of the whole of the genus of tumors to which the claims are directed" (page 10 of the pending Office Action). The Examiner is further of the opinion that

the combination of TaxolTM and cisplatin is not representative of the whole of the genus of agents to which the claims are directed. Because of the different structure, specificities, modes of action, additive and/or synergistic effects of different agents, the presence of one or more markers in any given tumor may not always correlate with its sensitivity to the agents (page 10-11 of the pending Office Action).

As an initial matter, Applicants point out that, in the interest of expediting prosecution and allowance of the pending claims, and in no way acquiescing to the validity of the Examiner's rejection, the claims have been amended such that they are now directed to ***ovarian cancer*** and an agent selected from the group consisting of: ***TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs, and cisplatin derivatives.***

Applicants respectfully traverse the foregoing rejection as it applies to the amended claims for the following reasons. Applicants submit that, based on the teachings in Applicants' specification and the knowledge generally available in the art, one of skill in the art would reasonably conclude that Applicants were in possession of the claimed invention at the time of filing. Written description may be satisfied through disclosure of relevant identifying characteristics, *i.e.*, structure, other physical and/or chemical characteristics, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." *Guidelines for Examination of Patent Applications Under the 35 U.S.C. §112, First Paragraph Written Description Requirement.* In the present case, Applicants describe identifying characteristics of the members of these families, including the structure of analogs, derivatives, and mimics that are structurally or functionally similar to TAXOL or cisplatin (see page 66, line 3 through page 70, line 24 of the specification). In particular, the present specification teaches that TAXOL and cisplatin are chemical compounds within the family of taxane and platinum compounds, respectively, which are art-recognized as families of *related* compounds (page 66, lines 4-5; and page 69, line 34 through page 70, line 1 of the specification). Numerous TAXOL analogs, derivatives and mimics are known in the art and are described in the instant specification, including Taxotere (docetaxol), which is described in U.S. Patent No. 5,840,929, and has been found to have good tumor activity in animal models (see page 66, lines 22-24 of the specification). In addition, Applicants teach in the specification how to generate TAXOL analogs, derivatives and mimics (having the formulas described at pages 67-69 of the specification) and incorporate by reference numerous patents which describe such analogs, derivatives and mimics in greater detail (see, for example, U.S. Patent Nos. 5,840,929; 5,773,464; 5,821,263; 4,814,470; 4,960,790; and 4,942,184). Applicants also teach methods for making TAXOL analogs, derivatives and mimics by, for example, the well established paclitaxel chemistry (page 68, lines 3-21 of the specification). Similarly, the instant specification teaches cisplatin analogs, derivatives and mimics (page 69, line 34 through

page 70, line 24 of the specification) and identifies and incorporates by reference numerous patents that describe such cisplatin analogs, derivatives and mimics (see page 70, lines 15-18 of the specification).

In addition to the foregoing teachings in Applicants' specification, the art at the time of the invention is replete with teachings regarding such compounds. As evidenced by, for example, U.S. Patent Nos. 5,840,929, 5,773,464, 5,641,803, 5,665,671, 5,380,751, 5,728,687, 5,415,869, 5,407,683, 5,399,363, 5,424,073, 5,157,049, 5,773,464, 5,821,263, 5,840,929, 4,814,470, 5,438,072, 5,403,858, 4,960,790, 5,433,364, 4,942,184, 5,362,831, 5,705,503, and 5,278,324 (the contents of each of which were expressly incorporated by reference in the present specification), such TAXOL analogs, derivatives and mimics were well known in the art at the time of the invention. Moreover, as evidenced by, for example, U.S. Patent Nos. 6,001,817, 5,945,122, 5,942,389, 5,922,689, 5,902,610, 5,866,617, 5,849,790, 5,824,346, 5,616,613, and 5,578,571 (the contents of each of which were expressly incorporated by reference in the present specification), such cisplatin analogs, derivatives and mimics were also well known in the art at the time of the invention.

Based on the foregoing teachings in Applicants' specification and the teachings in the art at the time of the invention, one of skill in the art would reasonably conclude that Applicants were in possession of the claimed methods at the time of filing. Accordingly, Applicants respectfully request that the aforementioned rejection be reconsidered and withdrawn.

The Examiner has also asserted that "the specification does not describe a genomic DNA molecule (*i.e.*, a gene) corresponding to the nucleotide sequence identified as SEQ ID NO:16...and does not teach whether the nucleotide sequence encodes all or part of a protein" (page 11 of Office Action). Applicants respectfully submit that, contrary to the Examiner's assertions, all that the claims require is determination of the expression of a marker of the invention, such as, for example, the marker of SEQ ID NO:16. Thus, a description of the corresponding genomic sequence is not required for the practice of the claimed invention. Based on the nucleotide sequence of a marker of the invention, such as, for example, the marker of SEQ ID NO:16 (depicted in Tables 2A-2B as "jlhbab412e01"), one of skill in the art would be able to

design probes or primers suitable for detecting the expression of this marker (as described at, for example, page 15, lines 30-35 and page 17, lines 12-19 of the specification).

Moreover, as indicated by the Court in *In re Deuel*, "cDNA contains only the protein-encoding regions of DNA. Thus, once a cDNA's nucleotide sequence is known, the amino acid sequence of the protein for which it codes may be predicted using the genetic code relationship between codons and amino acids." *In re Deuel*, 34 USPQ2d 1210, 1215 (Fed. Cir. 1995). Indeed, as is well known in the art, the amino acid sequence corresponding to a nucleic acid sequence may be easily determined by identifying the open reading frame or translating the sequence in all three reading frames. Such a determination may be made using well known programs designed, for example, to identify open reading frames and/or translate the nucleotide sequence in all frames. Therefore, based on the disclosure of the nucleotide sequences of the markers of the invention and the teaching in the specification that these sequences represent expressed products, one of skill in the art would conclude that Applicants were in possession of the corresponding protein sequence at the time of filing. Accordingly, Applicants respectfully request that the aforementioned rejection be reconsidered and withdrawn.

Rejection of Claims 1, 4-8, and 11-14 Under 35 U.S.C. §112, First Paragraph

The Examiner has also rejected claims 1, 4-8 and 11-14 under 35 U.S.C. §112, first paragraph as failing to comply with the enablement requirement. According to the Examiner, "the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims." As a first matter, the Examiner contends that "...the presence of one or more markers in different members of the genus of tumors may not always correlate with their sensitivity to an agent." The Examiner is further of the opinion that "...the combination of TaxolTM and cisplatin is not representative of the whole of the genus of agents to which the claims are directed."

As an initial matter, Applicants respectfully submit that in the interest of expediting prosecution, and in no way acquiescing to the validity of the Examiner's rejection, the claims have been amended such that they are now directed to *ovarian cancer* and an agent selected from the group consisting of *TAXOL*, *TAXOL mimics*, *TAXOL analogs*, *TAXOL derivatives*, *cisplatin*, *cisplatin mimics*, *cisplatin analogs*, and *cisplatin derivatives*.

Applicants traverse the foregoing rejection as it applies to the amended claims and submit that the amount of direction and guidance disclosed in the specification is commensurate with the scope of the claims and sufficient to enable the skilled artisan to make and use the claimed methods using only routine experimentation. The present claims are directed to methods for determining whether an agent selected from the group consisting of TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs, and cisplatin derivatives can or cannot be used to reduce the growth of an ovarian tumor. The claimed methods are not methods for the treatment of ovarian cancer. ***Thus, the appropriate inquiry for determining whether the enablement requirement has been satisfied in the present case, is whether the instant specification teaches the ordinary skilled artisan how to assess the expression of the markers of the invention, such as SEQ ID NO:16, in the patient sample and make a determination of whether to proceed with a therapeutic agent (i.e., an agent selected from the group consisting of TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs, and cisplatin derivatives) based on the foregoing assessment of expression in the patient sample.*** For the reasons set forth below, the instant specification enables the ordinary skilled artisan to perform the claimed methods.

It is well established that “[t]he examiner has the initial burden of establishing a reasonable basis to question the enablement provided for the claimed invention.” *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). A requirement for some experimentation does not prevent the satisfaction of the enablement requirement (*Northern Telecom, Inc. v. Datapoint Corp.*, 15 U.S.P.Q.2d 1321, 1329 (Fed. Cir. 1990)). The Federal Circuit has made it clear that “[w]hen rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, ***providing sufficient reasons for doubting any assertions*** in the specification as to the scope of enablement.” *In re Wright*, 999 F. 2d at 1561-1562 (Emphasis added).

Here, the PTO has not met this initial burden. Specifically, the Examiner has not provided any factual evidence to show a reason to doubt the objective truth of Applicants’

statements which must be relied upon for enabling support; namely, that the claimed methods may be used for determining whether an agent selected from the group consisting of TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs, and cisplatin derivatives can or cannot be used to reduce the growth of an **ovarian tumor that expresses the markers of the invention, e.g., the marker of SEQ ID NO:16**.

Instead, the Examiner cites Abuharbeid *et al.* (*Naunyn-Schmiedberg's Arch. Pharmacol.* 371:141-151 (2005)) for teaching the unpredictability of HER-2 expression in determining the sensitivity or insensitivity of cancer cells to an agent and for teaching the unpredictability of HER-2 expression in various types of cancer. Applicants respectfully point out that the findings of Abuharbeid *et al.* concern the expression of HER-2, not the claimed marker, SEQ ID NO:16. Moreover, the present claims are now directed to ovarian tumor cells, thus, the issue of conflicting data faced by Abuharbeid *et al.* is not relevant to the present case.

The Examiner further relies on Ayers *et al.* (*J. Clin. Oncol.*, 22(12):2284-2293 (2004)) for teaching that “no single marker [was] sufficient[ly] associated with [a] pathologic complete response to be used as an individual predictor.” However, Ayers *et al.* describe that the purpose of the study was to “examine the feasibility of developing a **multigene predictor** of pathologic complete response to sequential weekly paclitaxel and fluorouracil + doxorubicin + cyclophosphamide neoadjuvant chemotherapy regimen for breast cancer” (See “Purpose Section” on page 2284) (Emphasis added), not the identification of a single marker. Furthermore, Ayers *et al.* did not evaluate the expression of the claimed marker, SEQ ID NO:16, nor was the analysis performed in ovarian cancer tissue.

As evidenced by the foregoing, the teachings of Abuharbeid *et al.* and Ayers *et al.* are not relevant to the enablement of the claimed invention which is directed to methods of determining whether an agent selected from the group consisting of TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs, and cisplatin derivatives can or cannot be used to reduce the growth of an **ovarian tumor that expresses the markers of the invention, e.g., the marker of SEQ ID NO:16**. Certainly, one of ordinary skill in the art would expect, and Applicants' specification acknowledges, that “[t]he quality of being sensitive to a therapeutic agent is a variable one, with different tumors exhibiting different levels of ‘sensitivity’ to a given therapeutic agent, under different

conditions” (see page 6, lines 27-29 of the specification). At the same time, Applicants provide working examples which demonstrate that TAXOL and cisplatin are effective therapeutic agents against tumors that express the markers of the invention, including, for example, SEQ ID NO:16 (page 7, lines 26-33; page 65, line 34 through page 66, line 4; page 69, lines 33-34 of the specification; and Tables 2A and 2B). Moreover, Applicants’ specification teaches that TAXOL and cisplatin analogs, derivatives or mimics have the same therapeutic effect as TAXOL and cisplatin on ovarian tumors that express the markers of the invention, *e.g.*, the marker of SEQ ID NO:16 (see page 66, line 70 of the specification). As outlined above, the present specification also teaches TAXOL and cisplatin analogs, derivatives and mimics and identifies and incorporates by reference numerous patents that describe such cisplatin analogs, derivatives and mimics. Indeed, cisplatin derivatives, analogs and mimics were well known in the art at the time of the invention as evidenced by the plethora of U.S. patents outlined above. Moreover, as evidenced by Guy, RK. *et al.*, *Chem Biol.*, 3 (12):1021-31 (1996), submitted herewith as Appendix A, methods for generating TAXOL and cisplatin derivatives, mimics and analogs were well-known in the art at the time of filing. In particular, Guy *et al.* teaches a method for generating bioactive taxol derivatives and states that such a method “should easily adapt to the production of any labeled taxoid of interest” (pg. 1028, left column under “Significance”). Indeed, Guy *et al.* prove that the skill in the art in 1996 was sufficiently advanced that an ordinary skilled artisan would have been able to have practiced the claimed invention without undue experimentation, because the generation and testing of TAXOL derivatives, mimics or analogs was well known in the art.

Applicants also teach well known assays for assessing whether the expression of a marker of the invention correlates with the effectiveness of treatment of an agent selected from the group consisting of TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs, and cisplatin derivatives. For example, the present specification teaches that by examining the expression of one or more markers of the invention in a sample of cancer cells, it is possible to determine which therapeutic agents or combination of agents to use as the appropriate treatment agents (page 70, line 38 through page 71, line 2 of the specification). Furthermore, the instant specification describes the methods by which a cancer patient receiving a treatment of TAXOL, for example, would have cancer cells removed and monitored for the expression of a marker (assays for assessing such expression, including

Western blots, immunoprecipitations, and immunofluorescence, are taught at page 56, line 36 through page 57, line 1 of the specification). If the expression level of a sensitivity marker remains substantially the same, the treatment of TAXOL would continue; however, a significant decrease in sensitivity marker expression, would demonstrate that the cancer may have become resistant to TAXOL and that another chemotherapy protocol should be initiated to treat the patient (page 71, lines 3-13 of the specification).

Moreover, Applicants' specification teaches numerous methods for detecting whether a tumor sample expresses a marker of the invention, as claimed (page 56, line 27 through page 62, line 19 of the specification). For example, Applicants teach *in vitro* techniques for detecting mRNA, including Northern hybridizations and *in situ* hybridizations (page 56, lines 32-36 of the specification). Applicants further disclose the use of hybridization probes for the purpose of identifying nucleic acid molecules that correspond to a marker of the invention, including nucleic acids which encode a polypeptide corresponding to a marker of the invention, and fragments of such nucleic acid molecules (page 15, lines 27-35 of the specification). In addition, Applicants teach techniques for the detection of a polypeptide, which corresponds to a marker of the invention, including Western blots, immunoprecipitations, and immunofluorescence (page 56, line 36 through page 57, line 1 of the specification).

Based on the foregoing teachings in Applicants' specification and the knowledge generally available in the art at the time of the invention, the ordinarily skilled artisan would be able to make and use the claimed invention using only routine experimentation. It would, therefore, be unreasonable to require Applicants to demonstrate a correlation between the expression of a marker the invention, such as SEQ ID NO:16, and the effectiveness of treatment for each and every TAXOL analog, mimic or derivative and/or every cisplatin analog, mimic or derivative.

The Examiner has further asserted that "...the algorithms and methodology used in the analysis performed have not been described, such that it would be possible to repeat such analyses using other agents and other types of cancer" (page 15 of the Office Action).

Applicants respectfully traverse this rejection and submit that the algorithms used by Applicants are not required to practice the claimed invention, because Applicants have already

identified markers of the invention whose expression is correlated with sensitivity or with resistance to therapeutic agents, such as TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs and cisplatin derivatives. Thus, all that is required for the practice of the claimed invention is obtaining a sample of ovarian tumor cells; determining whether the ovarian tumor cells express one or more sensitivity markers identified in Tables 1-6; and identifying that the agent can be used to reduce the growth of the ovarian tumor cells when the one or more sensitivity markers are expressed. As indicated above, the instant specification provides ample guidance as to how one of skill in the art would assess the expression of a marker of the invention, such as, for example, SEQ ID NO:16, in a patient sample and make a determination of whether to proceed with a therapeutic agent (*e.g.*, an agent selected from the group consisting of TAXOL, TAXOL mimics, TAXOL analogs, TAXOL derivatives, cisplatin, cisplatin mimics, cisplatin analogs and cisplatin derivatives) based on the foregoing assessment of expression in the patient sample (see, for example, page 6, lines 13-21; page 14, line 19 through page 15, line 12; page 55, lines 32-37; page 56, line 32 through page 57, line 1; and page 70, line 30 through page 71, line 13 of the specification).

Assuming, *arguendo*, that the algorithms used by Applicants would be necessary for one of skill in the art to practice the claimed invention (which they are not), Applicants respectfully submit that the algorithms and methodology used were well known in the art at the time of the invention. For example, such regression models, known in the art at the time of filing, included Perl Object Oriented Framework (POOF) (see, for example, PCT Publication No. WO 01/42792), CART (*e.g.*, Hill, T, and Lewicki, P. (2006) "STATISTICS Methods and Applications" StatSoft, Tulsa, OK), Cox (*e.g.*, www.evidence-based-medicine.co.uk), exponential, normal and log normal (*e.g.*, www.obgyn.cam.ac.uk/rmrg/statsbook/stsurvan.html; see also, www.obgyn.cam.ac.uk/rmrg/statsbook/index.html); logistic (*e.g.*, www.en.wikipedia.org/wiki/Logistic_regression), parametric, non-parametric, linear (*e.g.*, www.en.wikipedia.org/wiki/Linear_regression), or additive (*e.g.*, www.en.wikipedia.org/wiki/Generalized_additive_model).

In summary, Applicants submit that based on the teachings in Applicants' specification and the knowledge generally available in the art at the time of the invention, the skilled artisan would have been able to make and use the claimed invention using only routine experimentation.

Accordingly, Applicants respectfully request that the aforementioned rejection be reconsidered and withdrawn.

CONCLUSION

In view of the above amendments and remarks, Applicants believe that the pending claims are in condition for allowance.

Please charge any fees associated with the filing of the present communication to our Deposit Account No. 12-0080, under Order No. MRI-027, from which the undersigned is authorized to draw.

Dated: April 6, 2006

Respectfully submitted,

By 

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Fluorescent taxoids

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Background: Taxol* is a natural product produced by the Pacific Yew, *Taxus brevifolia*, that has emerged as a prominent chemotherapeutic agent for the treatment of solid tumors. Taxol's biochemical mode of action has been well studied: it binds to microtubules, stabilizing them and preventing their depolymerization to tubulin subunits. At lower dosage levels, taxol also interferes with the normal dynamics of the tubulin-microtubule equilibrium. This biochemical effect causes taxol's ultimate physiological effect, cell cycle arrest; taxol is thought to block anaphase A of mitosis. Taxol also causes a number of intriguing secondary effects on interphase cells that are poorly understood. We believed that a bio-active fluorescent taxol derivative could be a useful tool in the study of these cellular mechanisms, especially in interphase cells.

Results: We have synthesized and characterized a series of stable, fluorescently labeled derivatives of taxol that bind to microtubules and have cytotoxicities similar to that of taxol. Fluorescence microscopy experiments in interphase human foreskin fibroblast (HFF) cells indicate that one of these, a sulforhodamine taxoid, is particularly well suited for optical microscopy. The use of this taxoid in HFF cells revealed a previously undetected localization of taxoids to the nucleolus during interphase.

Conclusion: The production of a new fluorescent derivative of taxol provides a useful tool, enabling cellular biologists to study taxol's mechanism of action. It is hoped that this material will prove particularly useful for the study of taxol's effects upon interphase cells.

Introduction

Taxol [1,2] (compound 1, Fig. 1) is an anticancer chemotherapeutic agent that was originally isolated in the late 1960's by cytotoxicity-directed fractionation of the methanolic extracts of the bark of the Pacific yew, *Taxus brevifolia* [3]. Although initial studies indicated that the new drug had considerable potential, development was delayed until the 1980's by the scarcity of the natural compound and its apparent lack of advantages over existing treatments [4]. The combination of early preclinical work at the National Cancer Institute (NCI) and the disclosure that taxol had an unusual biochemical mechanism (stabilization of microtubules) by Horwitz and coworkers [5,6], spurred on what became very rapid progression to taxol's current status as a widely used clinical agent. Early on in the clinical trials, researchers observed that the drug had an unusual ability to repress both breast [7] and ovarian [8] cancers. The drug was also shown to have significant effects on several other types of solid tumors including non-small-cell lung and head-and-neck cancers [9].

The study of taxol's mechanism of action has shed light upon the normal process of microtubule dynamics and

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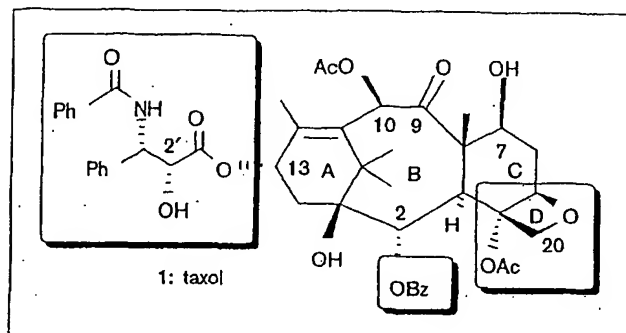
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gives insight into the importance of this process in cellular events [10]. Microtubules are crucial cellular structures. They are composed of heterodimeric tubulin subunits that polymerize to form the multisubunit microtubule, a helical linear polymer 25 nm in diameter with a central 15 nm hole. [11,12]. Normally, the microtubules of a cell are in a constant state of flux, growing and shrinking in a manner that has been characterized as 'dynamic instability' [13,14]. The dynamic properties of microtubules are central to many cellular processes, particularly the assembly and function of a mitotic spindle during cell division [15]. Taxol stabilizes the microtubule, preventing 'catastrophe' (the rapid depolymerization of the microtubule to free tubulin subunits) and reducing the amplitude and rate of the changes in microtubule length that are collectively known as 'microtubule dynamics' [16,17].

In cells, the biochemical effects of taxol ultimately lead to a strong inhibition of eukaryotic cell proliferation, usually blocking cell-cycle progression at the G₂/Pro transition into mitosis [18-20]. At this time, the interphase cytoskeleton is disassembled and the microtubules are reorganized into a bipolar array characteristic of the mitotic

*Although in common usage for the last 20 years, Taxol is now a registered trademark of Bristol-Myers Squibb. The copyright of Bristol-Myers Squibb is recognized when Taxol or taxol is used in this article.

Figure 1



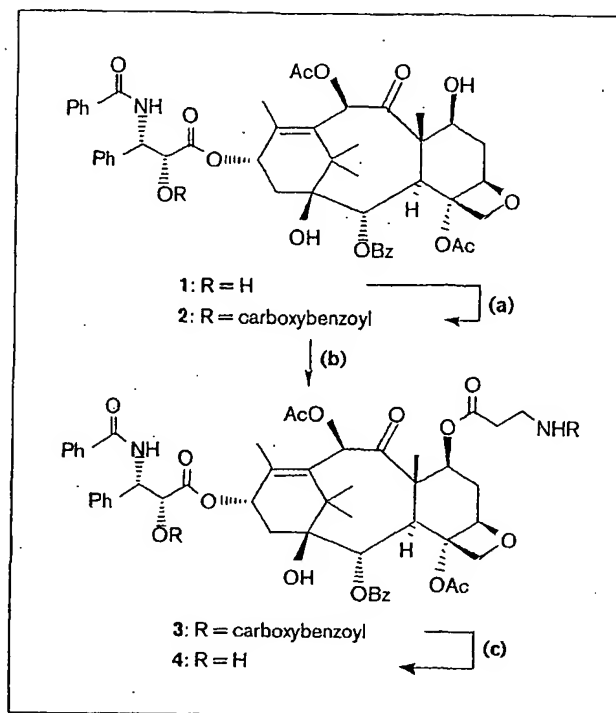
Structure of taxol, compound 1. The boxed substituents are crucial for biological activity. In reactions with electrophiles, the alcohol at the C2' position is the most reactive and that at C7 is the second most reactive.

apparatus. The drug also causes a number of cellular morphological changes in interphase cells [21–25], the most obvious of which is the formation of unusual bundles of microtubules [26–28]. Often, these studies are carried out using optical fluorescence microscopy with either anti-tubulin immunofluorescence or fluorescently labeled tubulin. These techniques require fixation and permeabilization or microinjection, respectively. We envisaged that a bioactive fluorescently labeled derivative of taxol, able to cross the cell membrane, would facilitate the use of fluorescence microscopy techniques. We also expected that such a taxoid would allow the study of previously unprobed areas, such as taxol localization in cells or the presence of a secondary receptor for the drug. In particular, we hoped that such a compound would stimulate work aimed at understanding taxol's effects upon the interphase portion of the cell cycle.

Before this project was begun, no fluorescent derivatives of taxol suitable for fluorescence microscopy had been reported. Kingston [29] had briefly mentioned, with little experimental detail, the production of a C-7 conjugated dansyl derivative of taxol, and Georg and coworkers [30] had reported the production of a sidechain analog of taxol, substituting the weakly fluorescent *m*-dimethylaminobenzoyl group for the normal benzoyl at the C-3'-nitrogen, and its use to study the polarity of the binding site microenvironment. Very recently two groups have reported the preparation of fluorescently labeled taxol [31] and taxotere [32] analogs designed for microscopy.

The design of our targeted derivatives and choice of a synthetic pathway to these compounds followed the outline shown in Figure 2. To allow us to choose the best fluorophore, and to provide a method for linking other spectroscopically active groups (such as those used to enhance magnetic resonance imaging (MRI) and positron emission spectroscopy (PET)), we wished to make all of

Figure 2

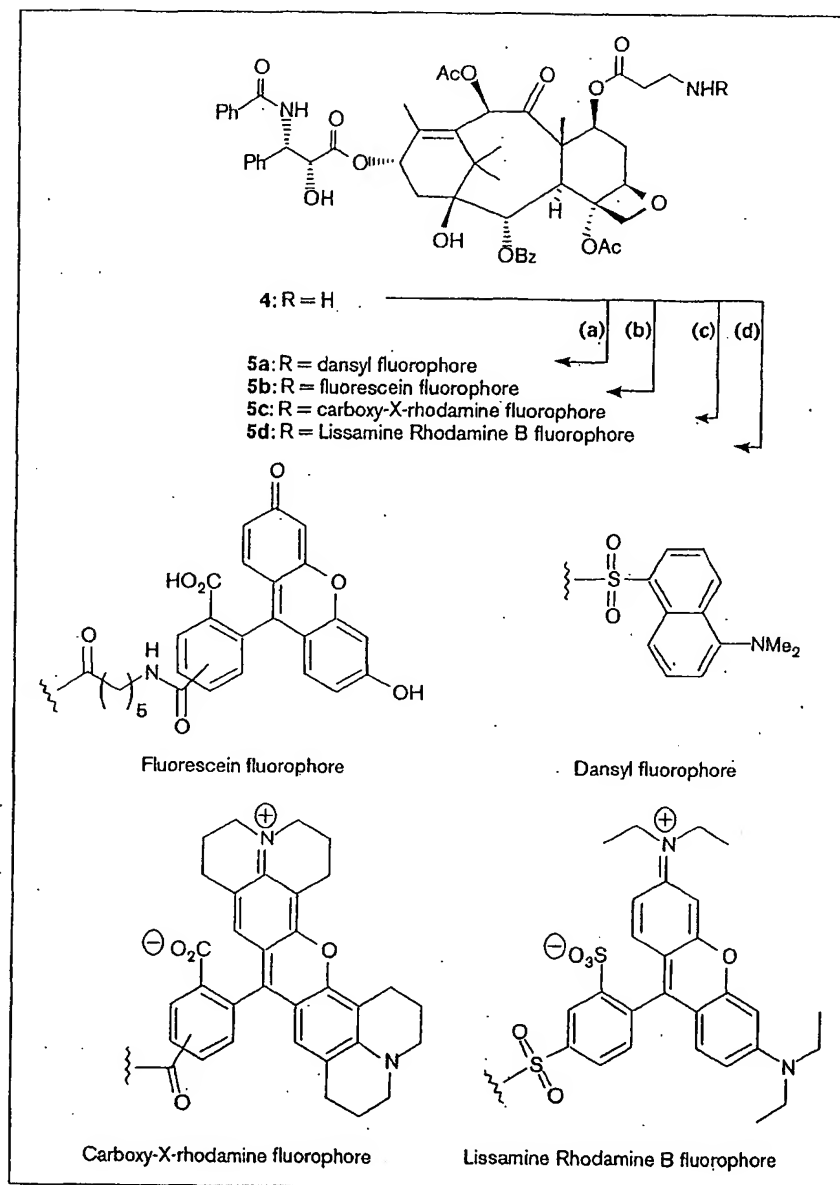


Synthesis of the common reactive intermediate 4. Reagents and conditions: (a) benzyl chloroformate (60 eq. in 10 eq. portions), pyridine (110 eq.), methylene chloride, 25 °C, 70 %; (b) N-CBz-β-alanine (4.4 eq.), dicyclohexylcarbodiimide (4.4 eq.), 4-(dimethylamino)pyridine (1 eq.), 25 °C, 18 h, 95 %; (c) H₂, 10 % Pd(C) (5 wt. %), MeOH, 25 °C, 2 h, quant.

the desired derivatives using a common reactive intermediate, containing an easily conjugated center. We therefore considered taxol's structure–activity relationship (SAR) [1,2,33] and its known patterns of reactivity. In general, modifications to the sidechain and to the substituents on the 'southern' portion (see boxed regions, Fig. 1) of the molecule are poorly tolerated while changes to the 'northern' portion have little effect upon the drug's activity (see unboxed regions, Fig. 1). Derivatization of the OH group at C-7 of the C-ring of the taxane nucleus to form an ester is usually well tolerated [34–36]. The reactivity of taxol with electrophiles has been well studied [1,2]. The C-2'-hydroxyl of the sidechain is normally the most reactive and can be readily differentiated from the other hydroxyls. When C-2' is protected, reaction usually proceeds selectively at the C-7 hydroxyl. It was therefore decided to derivatize the C-7 hydroxyl of taxol with an ester that contained a nascent pendant amine. Deutch's group [37] has shown that α-amino esters of this alcohol are fairly unstable. We therefore decided to try to use the β-amino alanine ester as a general linker. To maximize the probability of producing a functional fluorescent taxoid, we explored several different conjugating groups and

Figure 3

Synthesis of fluorescent taxoids 5a-d. Reagents and conditions: (a) dansyl chloride, triethylamine, acetonitrile, 25 °C, 10 min, 69 %; (b) fluorescein N-hydroxysuccinate ester, 10 % aqueous sodium bicarbonate, dioxane, 25 °C, 1.2 h, 65 %; (c) carboxy-X-rhodamine N-hydroxysuccinate ester, 10 % aqueous sodium bicarbonate, dioxane, 25 °C, 1.5 h, 73 %; (d) Lissamine Rhodamine B chloride, 10 % aqueous sodium bicarbonate, dioxane, 25 °C, 2 h.



tether lengths for the fluorophores. Because the presence of mixed isomers at the fluorophore's aromatic nucleus does not affect the function of the material in fluorescence microscopy, two of the fluorophores (fluorescein and carboxy-X-rhodamine) were introduced from the commercially available mixture of isomers.

Results and discussion

Synthetic chemistry

With the synthetic goals in mind, taxol (compound 1, Fig. 1) was reacted with benzyl chloroformate in the presence of pyridine to give the 2'-benzyl carbonate (compound 2, Fig. 2) of taxol in good (70 %) yield. This

was then acylated with N-carboxybenzyl- β -alanine by treating the alcohol with dicyclohexylcarbodiimide and 4-(N,N-dimethylamino)-pyridine to give the ester 3 (Fig. 2) in high (95 %) yield. Concomitant reductive removal of both carboxybenzyl protecting groups gave the desired amine 4 (Fig. 2) in essentially quantitative yield. This amine was then conjugated with several fluorophores in good (65–73 %) yield to give the fluorescently labeled derivatives 5a–d (Fig. 3).

As mixed isomers pose no problems for fluorescence microscopy, we did not separate the aromatic isomers of the fluorescein and carboxy-X-rhodamine conjugates. The

Table 1

Physical properties of the fluorescent taxoids 5a-d.

	1	5a	5b	5c	5d
Aqueous solubility (M)	$<< 1 \times 10^{-6}$	$<< 1 \times 10^{-6}$	4×10^{-5}	2×10^{-5}	1×10^{-5}
Partition coefficient (n-octanol/water)	> 50	> 50	1	2	2
Fluorescence maxima (nm, excitation/emission)	—	350 / 500	512 / 550	400 / 610 550 / 610	380 / 620 525 / 620

β -alanyl linker proves to be a good 'handle' for conjugation of taxol. The method described above is efficient enough (43–48 % overall yield from taxol) that it is possible to make fluorescent taxoids readily available in any reasonably anticipated quantities for most purposes. The overall method should be easily adaptable to produce other taxol conjugates.

Physical properties

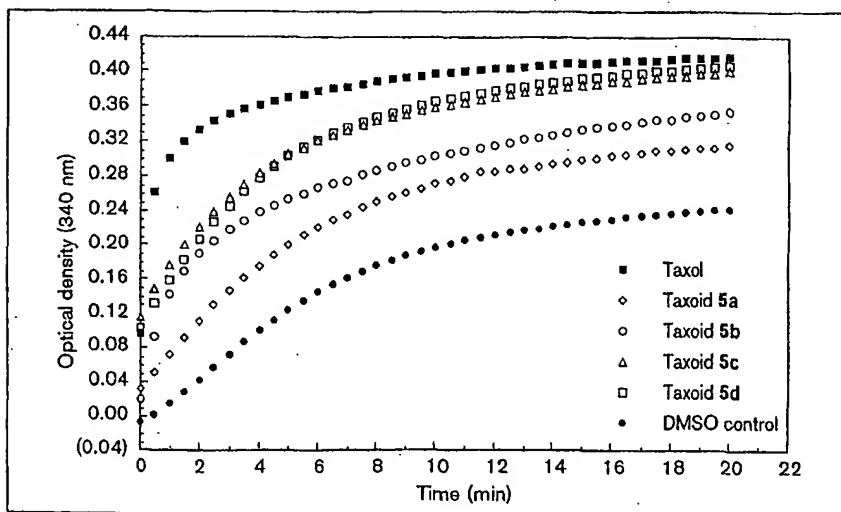
The solubility, stability, and spectroscopic characteristics of the fluorescently labeled taxoids were examined to determine their suitability for further study (Table 1). UV spectroscopic assays show that the three derivatives that contain formal charges, compounds 5b–d, are significantly more soluble in water than taxol. The dansyl taxoid 5a retained taxol's highly lipophilic character. The partition coefficients (octanol to water) exhibited a similar shift towards hydrophilic character for the charged derivatives 5b–d. All the conjugates were reasonably stable in solution (>14 days at room temperature in ethanol (1 mM) with no apparent degradation). Finally, all conjugates retained reasonable excitation/emission spectra (see Table 1) allowing the use of standard filter blocks for fluorescence

microscopy. An interesting characteristic of the rhodamine labeled taxoids 5c and 5d is that they developed a secondary excitation wavelength after conjugation to the taxoid nucleus. The combination of these characteristics was sufficiently promising to warrant further study.

Tubulin polymerization assays

To test whether the addition of fluorescent groups had impaired the activity of our modified taxoids, we examined their ability to polymerize chick brain tubulin *in vitro* (Fig. 4) [38]. The tubulin used in these assays retained the microtubule-associated proteins (MAPs) [39,40]; this system was viewed as more physiologically relevant. All the taxoids showed enhanced rates of tubulin polymerization and a higher plateau for bulk polymerization levels (as determined when the change of the absorbance reached the level of +0.002/–0.004 A units per min) than the DMSO control. Among the derivatives, the two rhodamine taxoids 5c and 5d exhibited the best tubulin polymerization profiles, equaling that of the taxol control within experimental error ± 0.002 OD. The fluorescein labeled taxoid 5b had the next best tubulin-polymerizing ability and the dansyl derivative was least promising.

Figure 4



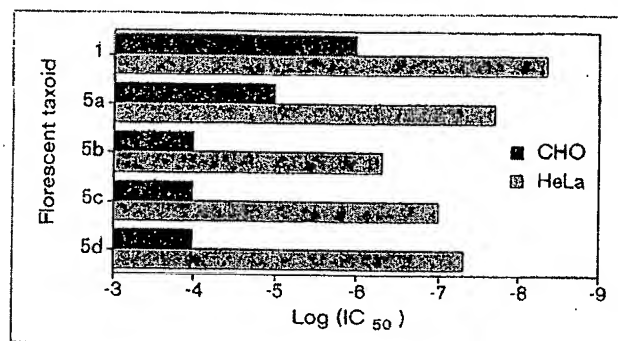
Relative tubulin polymerization abilities of taxol and the fluorescent taxoids 5a–d. The bulk polymerization was determined by following the change in optical density at 340 nm spectroscopically. For particulars, see Materials and methods.

These results correlate directly with the partition coefficients and aqueous solubility of the respective derivatives, perhaps indicating that profitable interaction with the microtubule requires a group on C-7 that retains the ability to hydrogen bond with bulk water. The observation that these derivatives are slightly less active than taxol is not unexpected, given previous results with other derivatizations at this position [32]. All of the fluorescent taxoids retain enough binding to microtubules that it is reasonable to expect them to be active *in vivo*.

Cytotoxicity assays

To evaluate how well the fluorescently labeled taxoids retained their cytotoxicity, the derivatives were tested for their ability to inhibit the growth [41] of two cell lines: Chinese hamster ovary (CHO) and HeLa cervical carcinoma (HeLa) (Fig. 5). All the derivatives had significant entotoxic effects, though attenuated relative to taxol. This trend held for both cell lines, although the magnitude of growth inhibition was much lower in the CHO line (as expected since this cell line is normally less sensitive to taxoids than HeLa). The dansyl taxoid, 5a, showed the highest cytotoxicity. The two rhodamine derivatives (5c and d) had approximately equal cytotoxicities that were

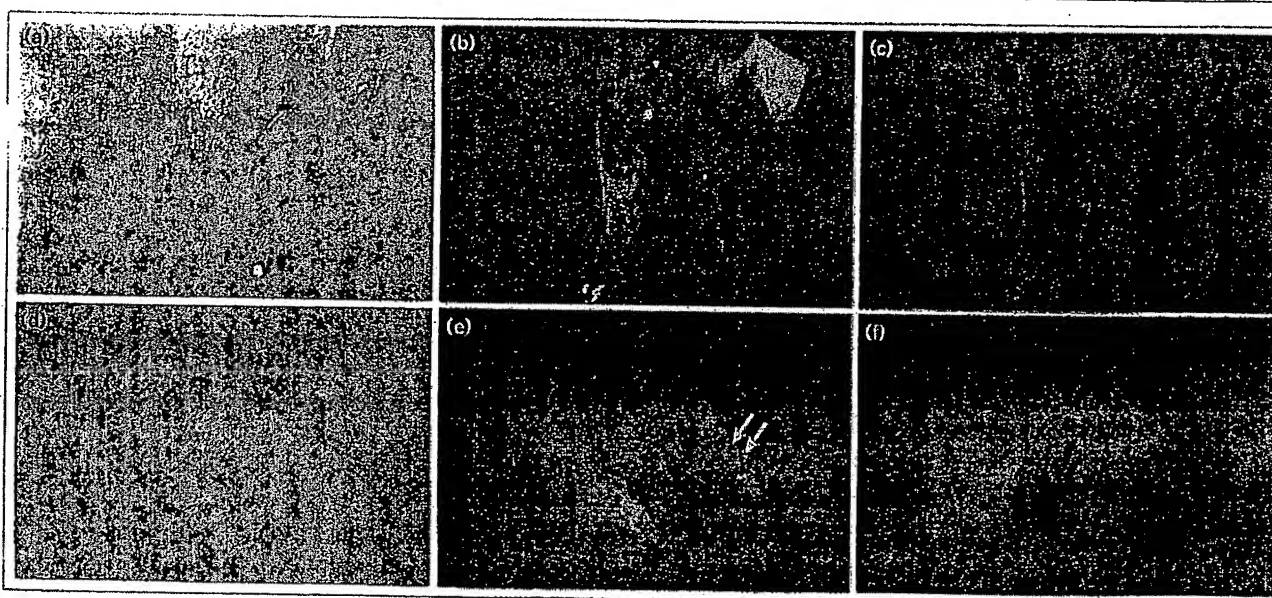
Figure 5



Relative cytotoxicities of taxol (1) and the fluorescent taxoids 5a-d. The cytotoxicities were determined using the standard XTT assay. For particulars, see Materials and methods.

less than that of the dansyl taxoid, and the fluorescein derivative had the lowest cytotoxicity of the four. These results can not be directly related to either partition coefficient or aqueous solubility. All the charged derivatives have lower cytotoxicities than the uncharged derivative or the taxol control, however. This may be because the

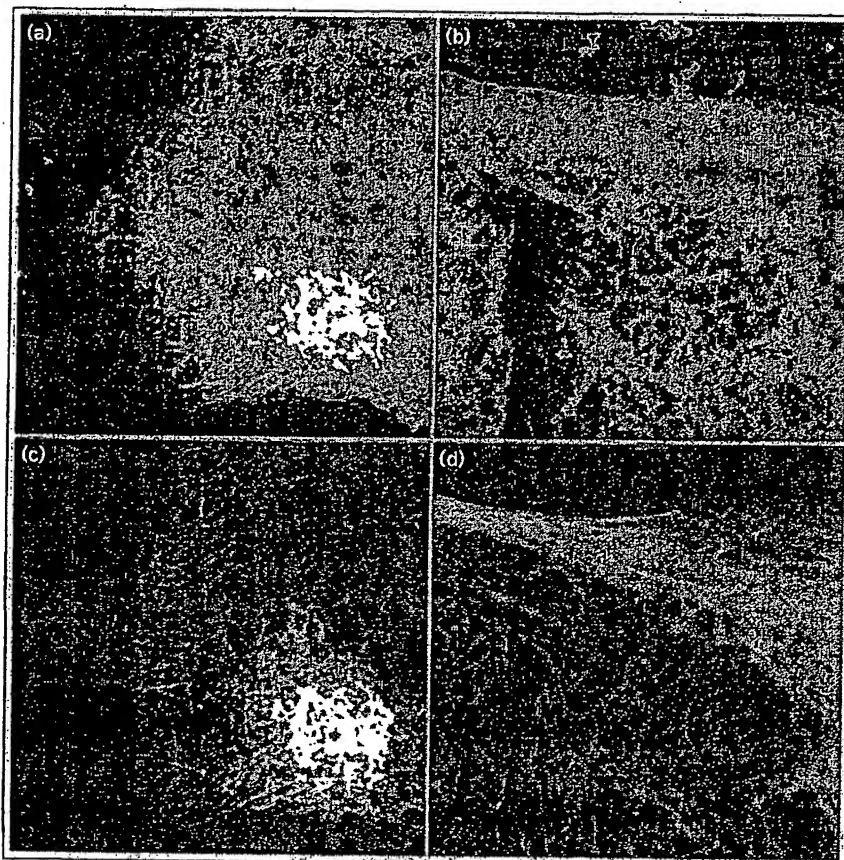
Figure 6



Microscopy images of human foreskin fibroblasts after exposure to Lissamine Red taxoid 5d (all cells have been fixed to allow comparison with the controls). (a) DIC photomicrograph showing normal cellular morphology after 1 h exposure to the drug (20 μ M, 37 $^{\circ}$ C). (b) Fluorescence photomicrograph of the same field shown in (a), using the rhodamine filter set, showing the fluorescence signal of the taxoid 5d. (c) Fluorescence photomicrograph of the same field shown in (a), using the fluorescein filter set, showing the microtubule cytoskeleton, as illuminated by traditional immunofluorescence using an α -tubulin monoclonal. (d) DIC photomicrograph showing the abnormal cellular

morphology that develops after 24 h exposure to taxoid 5d (20 μ M, 37 $^{\circ}$ C). (e) Fluorescence photomicrograph of the same field shown in (d) showing the fluorescence signal of the taxoid. Note the small structures of localized fluorescence (nucleoli marked by arrows) that do not appear in the immunofluorescence control. (f) Fluorescence photomicrograph of the same field shown in (d) showing the microtubule cytoskeleton by traditional immunofluorescence after 24 h exposure (note the absence of nucleoli). Clearly, in general the fluorescent taxoid reveals the same cytoskeletal structures as does traditional immunofluorescence. For experimental details, see Materials and methods.

Figure 7



Confocal microscopy images of human foreskin fibroblasts after 1 h exposure to Lissamine Red taxoid 5d (20 μ M, 37 $^{\circ}$ C, cells were fixed to allow comparison to the controls). The instrument was configured for blue to far red fluorescence excitation (458, 568 and 647 nm) enabling three-color confocal imaging of cells directly. (a) The rhodamine signal. Note the high degree of detail revealed by the Lissamine taxoid 5d relative to the controls. (b) Electronic magnification (2 \times) of a portion of (a) showing the fine details that are visualized by taxoid 5d. (c) Fluorescence photomicrographs of the same field shown in (a), using the fluorescein filter set, showing the fluorescence signal from a traditional immunofluorescence α -tubulin monoclonal. (d) Electronic magnification (2 \times) of a portion of (c). For experimental details, see Materials and methods

charged derivatives diffuse less well across the hydrophobic cell membrane, as observed previously with other charged derivatives at this position [42]. These results, combined with those from the experiments described above, clearly indicated that the rhodamine derivatives are the most promising for microscopy.

Fluorescence microscopy

We performed fluorescence microscopy experiments with three taxoids, the two rhodamine-labeled taxoids and the fluorescein-labeled one. These studies used cultured human foreskin fibroblasts, chosen because they become quiescent due to contact inhibition when well spread on the cover slips. In this state they have a clearly visible interphase array of microtubules. They therefore provide a good model for interphase cells, which these taxoids were designed to study. The fluorescein taxoid 5b exhibited rapid photobleaching and was not pursued further. Both the carboxy-X-rhodamine 5c and Lissamine 5d taxoids had reasonable amplitudes of fluorescence and fluorescence lifetimes. Most of the microscopy studies were carried out using the Lissamine-labeled taxoid, because it has a slightly higher tubulin polymerization ability and cytotoxicity, a slightly cleaner fluorescence

appearance, and the starting material for its chromophore is relatively inexpensive.

When the cells were treated with fluorescent taxoid 5d, they began to display fluorescent cellular networks of microtubules after \sim 10 min (Fig. 6b), a rate approximately equal to that with which fluorescently labeled tubulin is incorporated into the cytoskeleton. The micrographs shown in Figures 6 and 7 depict fixed cells to allow comparison between the fluorescent taxoid and the immunofluorescence control. Similar images can be obtained with living cells (see Fig. 8). Double label fluorescence studies using the fluorescent taxoid and indirect immunofluorescence with anti- α -tubulin primary antibodies showed that the derivatives illuminate the same microtubule skeleton as does traditional immunofluorescence (Fig. 6c), and that the derivative does not block the binding of these antibodies to microtubules. The level of signal from the fluorescent taxoid 5d is higher than that from the antibody controls (see Fig. 7). The most likely explanation for this is that, under the experimental conditions employed, the taxoid (at 20 μ M) has saturated the binding sites of the microtubules (previously estimated at 10–20 μ M, E.D. Salmon, personal communication) while the anti- α -tubulin

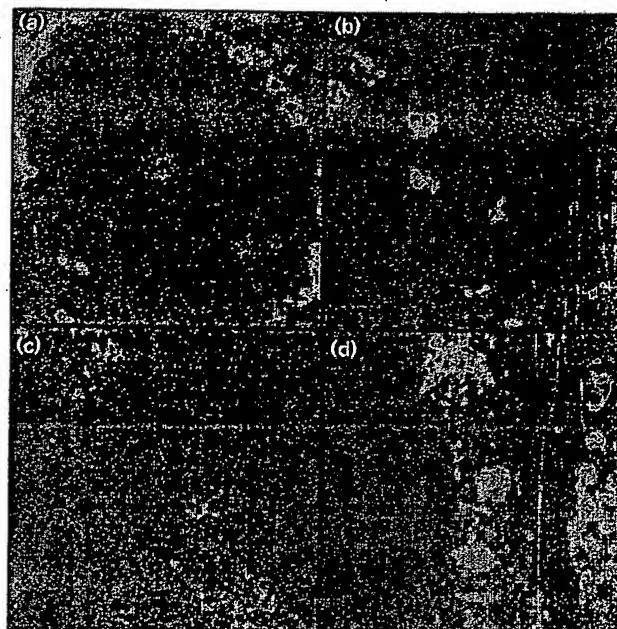
antibody has not and therefore gives a weaker signal. There is also some evidence of a diffuse background signal that may represent unbound taxoid or agent bound to small tubulin oligomers. Given the degree of overall tubulin polymerization demonstrated by the Lissamine taxoid 5d *in vitro*, it is more likely that the background signal is due to small oligomers. Thus, the net result of visualization with Lissamine taxoid 5d is a more richly detailed image than that provided by immunofluorescence. It is important to note that, unlike immunofluorescence images, these images can be obtained from living cells.

Cells treated with fluorescent taxoids retained normal morphology (Fig. 6a) and microtubule networks (Fig. 6b) for ~90–120 min, after which the cells appeared progressively abnormal by differential interference contrast (DIC) microscopy. After 20 h of treatment with the fluorescent derivative, the long extensions of the cells had retracted somewhat (Fig. 6d) and their microtubule networks appeared more bundled than filamentous (Fig. 6e). These results are as expected for a human cancer cell line treated with a taxoid. They demonstrate that the fluorescent conjugation of taxol does not significantly perturb the behavior of these derivatives in living cells. It is therefore reasonable to assume that such compounds could be used to study taxol's cellular mechanism of action. The synthetic chemistry described here is generally applicable to all taxoids containing a C-7 hydroxyl group, allowing this method of conjugation to be adapted to specific taxoids of interest. This class of derivatives should therefore enable workers to examine differences in the cellular behavior of specific taxoids with different physiological effects, especially in interphase cells.

In the cells that had been treated with taxoids 5c and 5d for an extended period of time, some of the taxoid appears to have partitioned into structures that do not include tubulin. These structures include nucleoli (see those marked by arrows in Fig. 6c and 8) and, occasionally, small punctate objects. Immunofluorescence controls revealed no co-localization of α -tubulin antibodies for either type of partitioning and therefore, if these structures contain microtubules the tubulin epitope must no longer be accessible to the α -tubulin antibody.

The punctate structures that occasionally form in the cytoplasm may be small asters of microtubules; these are microtubule structures that have been observed by DIC microscopy in taxol-treated sea urchin eggs [43]. A more likely explanation for this phenomenon, however, is that the punctate structures are inclusion bodies into which the taxoids have been segregated in an effort to reduce their toxic effects. Taxol is more effective at killing cancer cells *in vitro* when administered as a slow infusion over 6–24 h [44]. It may be that inclusion body formation explains this empirical observation; perhaps inclusion bodies form

Figure 8



Competition between unlabeled taxol 1 and Lissamine-labeled taxoid 5d for intracellular binding sites. The series of experiments shows that staining of the nucleoli is retained despite competition by unlabeled taxol. The figure depicts confocal microscopy images of human foreskin fibroblasts after 24 h exposure to a mixture of taxol 1 and Lissamine taxoid 5d (20 μ M overall taxoid concentration, held constant in all experiments, 37 $^{\circ}$ C, cells are living and unfixed). The instrument was configured for far red fluorescence excitation. (a) The rhodamine signal generated by 100 % Lissamine taxoid 5d. Note the staining of nucleoli (globular structures in the nucleus). (b) A 3:1 mixture of taxol 1 and Lissamine taxoid 5d. The relative level of signal between the microtubule based cytoskeleton and the nucleoli remains the same as in (a). (c) A 1:1 mixture of 1 and 5d. (d) A 1:3 mixture of 1 and 5d. The images in (c) and (d) were produced by photon accumulation and software control on the confocal microscope. This technique allowed the observation of the otherwise extremely weak overall fluorescence signal from the dilute Lissamine taxoid 5d. For experimental detail, see Materials and methods.

only when too much drug is delivered in a given period. Alternatively, the fluorophore itself could be causing the segregation. A final possibility could be that the punctate structures are not securely fixed and are washed away during the immunofluorescence staining.

The main fluorescence signal other than that due to tubulin distribution is the nucleolus which regularly exhibits fluorescence after exposure to 5d. There is no evidence for the presence of microtubules or tubulin in the nucleolus, but there is good evidence that one class of MAPs (the tau proteins) is present in the nucleolus of certain vertebrate tissue culture cells [45,46]. As the nucleolus is not a membrane-bound organelle, the fact that the relatively hydrophobic taxoid 5d accumulates within it cannot be explained by partitioning of the hydrophobic

taxane into a membrane. In competition experiments with constant (20 μ M) levels of total taxol and decreasing proportions of labeled taxol 5d the relative levels of signal from the nucleolus and cytoskeleton remained constant (see Fig. 6). This result indicates that the binding of taxoid 5d to the nucleolus is at least as specific as its binding to the microtubule cytoskeleton. There are two reasonable explanations for this observation: there may be previously undetected tubulin subunits in the nucleolus itself during interphase, or the nucleolus may contain a previously undetected receptor for taxol other than microtubules. As the evidence against the presence of microtubules in the nucleus is strong, the second explanation seems most plausible. The potential of a new receptor for taxol raises interesting possibilities for further study.

Significance

The work presented above shows that the C-7 position of taxol can be substituted with a variety of groups attached via a β -alanine ester linker. These studies have defined a general synthetic route to taxoids of this type that proceeds with good overall yield. The method should easily adapt to the production of any labeled taxoid of interest. According to these studies, substitutions at the C-7 position of the taxane nucleus retain reasonable biological activity. In this study, we made a bioactive fluorescent derivative of taxol, 5d, that is useful for fluorescence microscopy. This derivative allows study of the microtubule-based cytoskeleton of living cells without using invasive techniques such as the microinjection of fluorescently labeled proteins or the more severe fixation procedures required for immunofluorescence. The inherently high level of signal provided by using a large excess of a freely-diffusible specific fluorophore, such as 5d, provides a more detailed image than traditional immunofluorescence. It is hoped that these derivatives will prove useful for researchers who are examining the dynamic properties of microtubules in cells. In particular, we hope that these derivatives will enable studies of the effects of taxol in the relatively untouched area of interphase cells. These derivatives have already revealed the subcellular localization of taxoids to the nucleolus — an unanticipated and intriguing result.

Materials and methods

Chemistry

General techniques

All reactions were carried out in an argon atmosphere with freshly dried solvents under anhydrous conditions, unless otherwise noted. All reagents were purchased at highest commercial quality and used without further purification, unless otherwise stated. Preparative scale high performance liquid chromatography (HPLC) was carried out using a Vydak RP-18 (22.5 mm by 250 mm) column with a flow rate of 9 ml min⁻¹ and UV detection at 254 nm. NMR spectra were recorded on a Bruker AMX-500 instrument and calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used: s, singlet; d, doublet; t, triplet; m, multiplet; band, several overlapping signals; br, broad. The carbon numbering of taxol

was used to assign protons. Overlapping carbon signals were reported only once. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. High resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions.

2'-Carboxybenzoyltaxol 2

A solution of taxol (1) (100 mg, 0.117 mmol) and pyridine (950 μ l, 11.7 mmol, 100 equivalent (eq.)) in methylene chloride (5 ml) at room temperature under argon was treated, at 10 min intervals, with benzyl chloroformate (170 μ l aliquots, 1.17 mmol, 10 eq. to total of 60 eq.). When thin layer chromatography (TLC) (silica, 50 % EtOAc in hexanes, UV/PMMA) showed no remaining starting material, the reaction was quenched with aqueous NH₄Cl (1 ml). After dilution with ethyl acetate (10 ml), the reaction mixture was washed with ammonium chloride (2 \times 5 ml), copper sulfate (4 \times 10 ml), and sodium chloride (5 ml). The combined organic layer was dried (Na₂SO₄), concentrated, and purified by flash chromatography (silica, 50 % EtOAc in hexanes) to give 2'-carboxybenzoyltaxol as a white powder (80 mg, 70 %): R_f = 0.50 (silica, 50 % EtOAc in hexanes); T_g (glass transition) = 152–154 °C; melting point (mp) = 206–210 °C; $[\alpha]_D^{25}$ -53.8 (c 0.01 g ml⁻¹, CHCl₃); IR (thin film) ν_{max} 1240, 1371, 1724, 2962, 3511 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ , 1.14 (s, 3 H, 16-H), 1.25 (s, 3 H, 17-H), 1.69 (s, 3 H, 19-H), 1.76 (s, 1 H, 1-OH), 1.93 (s, 3 H, 18-H), 1.9–1.94 (m, 1 H, 6 β -H), 2.2–2.3, 2.4–2.5 (m, 2 H, 14-H), 2.24 (s, 3 H, 10-OAc-H), 2.46 (s, 3 H, 4-OAc-H), 2.51 (s, 1 H, 7-OH), 2.60 (m, 1 H, 6 α -H), 3.82 (d, J = 7, 1 H, 3-H), 4.20 (d, J = 8.5, 1 H, 20-H, A of AB), 4.32 (d, J = 8.5, 1 H, 20-H, B of AB), 4.41 (m, 1 H, 7-H), 4.98 (d, J = 8, 1 H, 5-H), 5.18 (dd, J = 9, 10, 2 H, Bn, AB), 5.45 (d, J = 2.5, 1 H, 2'-H), 5.69 (d, J = 7, 1 H, 2'-H), 5.98 (dd, J = 2.5, 9.5, 1 H, 3'-H), 6.30 (band, 2 H, 10-H, 13-H), 6.92 (d, J = 9.5, 1 H, NH), 7.30–7.42 (band, 12 H, Ar-H), 7.44–7.54 (band, 3 H, Ar-H), 7.61 (t, J = 7, 1 H, Ar-H), 7.73 (d, J = 8.5, 2 H, Bz-H), 8.14 (d, J = 8.5, 2 H, Bz-H); ¹³C NMR (125 MHz, CDCl₃) δ 9.5, 14.7, 20.7, 22.0, 22.6, 26.7, 35.4, 35.5, 40.5, 44.4, 45.4, 52.6, 58.2, 70.7, 71.9, 72.0, 74.9, 75.5, 76.3, 76.7, 78.1, 81.2, 84.3, 126.5, 127.0, 128.3, 128.4, 130.1, 131.9, 132.1, 133.0, 134.0, 137.2, 142.5, 154.1, 166.9, 167.2, 168.8, 171.1, 204.1; FAB HRMS (NBA/CsI) m/e found: 1121.2852 (M + Cs⁺); calc'd for C₅₅H₅₇NO₁₆Cs: 1121.2810.

2'-Carboxybenzoyl-7-(N-carboxybenzoyl- β -alanine)-taxol 3

A solution of 2'-carboxybenzoyltaxol 2 (25 mg, 0.025 mmol) and 4-(N,N-dimethylamino)-pyridine (3 mg, 0.025 mmol, 1 eq.) in methylene chloride (2 ml) at room temperature under argon was treated with N-carboxybenzoyl- β -alanine (24 mg, 0.11 mmol, 4.4 eq.) and dicyclohexylcarbodiimide (22 mg, 0.11 mmol, 4.4 eq.). After 18 h, the mixture was diluted with methylene chloride (5 ml) and filtered to remove the urea. The crude product was evaporated to dryness *in vacuo* and purified by flash chromatography (silica, 40–45 % EtOAc in hexanes) to give 2'-carboxybenzoyl-7-(N-carboxybenzoyl- β -alanine)-taxol as a white powder (28 mg, 95 %): R_f = 0.55 (silica, 50 % EtOAc in hexanes); mp = 114–116 °C; $[\alpha]_D^{25}$ -45.7 (c 0.01 g ml⁻¹, CHCl₃); IR (thin film) ν_{max} 1176, 1240, 1372, 1651, 1728, 1747, 2936, 3401 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ , 1.15 (s, 3 H, 16-H), 1.21 (s, 3 H, 17-H), 1.81 (s, 3 H, 19-H), 1.99 (s, 3 H, 18-H), 2.06 (s, 3 H, 10-OAc-H), 2.21 (m, 1 H, 6 β -H), 2.4–2.6 (band, 5 H, 14-H, 6 α -H, alanyl CH₂), 2.46 (s, 3 H, 4-OAc-H), 3.4–3.5 (m, 2 H, alanyl CH₂), 3.96 (d, J = 7, 1 H, 3-H), 4.20 (d, J = 8.5, 1 H, 20-H, A of AB), 4.33 (d, J = 8.5, 1 H, 20-H, B of AB), 4.95 (d, J = 8.5, 1 H, 5-H), 5.18 (dd, J = 9, 10, 2 H, Bn, AB), 5.45 (d, J = 2.5, 1 H, 2'-H), 5.63 (dd, J = 5.7, 1 H, 7-H), 5.70 (d, J = 7, 1 H, 2-H), 5.98 (dd, J = 2.5, 9, 1 H, 3'-H), 6.22–6.30 (band, 2 H, 10-H, 13-H), 6.98 (d, J = 9, 1 H, NH), 7.30–7.42 (band, 17 H, Ar-H), 7.43–7.52 (band, 3 H, Ar-H), 7.61 (t, J = 7, 1 H, Ar-H), 7.73 (d, J = 8.5, 2 H, Bz-H), 8.14 (d, J = 8.5, 2 H, Bz-H); ¹³C NMR (125 MHz, CDCl₃) δ 10.8, 14.4, 20.4, 21.3, 22.5, 24.8, 25.5, 26.3, 33.3, 33.8, 34.2, 35.3, 43.1, 46.5, 48.2, 52.7, 55.9, 66.3, 70.7, 71.4, 71.9, 74.4, 75.2, 76.6, 77.2, 78.5, 80.7, 83.7, 126.5, 127.0, 127.8, 128.0, 128.4, 128.6, 128.8, 128.9, 130.1, 131.9, 132.4, 133.4, 133.6, 136.6, 141.2, 153.9, 156.2, 166.8, 167.1, 167.8, 169.4, 169.6, 171.0, 202.1; FAB HRMS (NBA/CsI) m/e found: 1326.3553 (M + Cs⁺); calc'd for C₆₆H₆₈N₂O₁₉Cs: 1326.3549.

7- β -alanyltaxol 4

A solution of compound 3 (25 mg, 0.021 mmol) in methanol (1 ml) at room temperature under argon was treated with 10 % palladium on carbon (1 mg, 4 wt %) and placed under hydrogen. After 2 h, the mixture was filtered to remove the catalyst and evaporated to dryness *in vacuo* to give 7- β -alanyltaxol 4 as a white powder (19 mg, quant.): mp = 215–218 °C; R_f = 0.55 (silica, 10 % MeOH in methylene chloride); $[\alpha]_D^{24}$ –42.1 (c 0.01 g ml⁻¹, CHCl₃); IR (thin film) ν_{\max} 1176, 1240, 1412, 1662, 1716, 1765, 2990, 3301, 3482 cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ , 1.09 (s, 3 H, 16-H), 1.16 (s, 3 H, 17-H), 1.78 (s, 3 H, 19-H), 1.78–1.89 (m, 1 H, 6 β -H), 1.87 (s, 3 H, 18-H), 1.91–2.00 (m, 1 H, 14-H), 2.18 (s, 3 H, 10-OAc-H), 2.19–2.24 (m, 1 H, 14-H), 2.38 (s, 3 H, 4-OAc-H), 2.50–2.59 (m, 1 H, 6 α -H), 2.65–2.67 (m, 2 H, alanyl CH₂), 3.12–3.22 (m, 2 H, alanyl CH₂), 3.90 (d, J = 7, 1 H, 3-H) 4.19 (dd, J = 7, 14, 2 H, 20-H), 4.73 (d, J = 6, 1 H, 2'-H), 5.00 (d, J = 8.5, 1 H, 5-H), 5.63–5.65 (band, 3 H, 2-H, 3'-H, 7-H), 6.14 (br t, J = 6, 1 H, 13-H), 6.19 (s, 1 H, 10-H), 7.28–7.67 (band, 11 H, Ar-H, NH), 7.85 (dd, J = 1.5, 7.5, 2 H, Bz-H), 8.14 (dd, J = 1.5, 7.5, 2 H, Bz-H); ¹³C NMR (125 MHz, CD₃OD) δ 11.4, 14.7, 20.8, 22.1, 23.2, 25.4, 26.7, 32.4, 36.3, 36.5, 44.6, 47.9, 49.2, 57.2, 57.7, 72.1, 73.6, 74.9, 75.8, 76.9, 77.3, 78.8, 80.0, 81.8, 85.0, 128.5, 129.0, 129.6, 129.7, 131.2, 132.9, 134.1, 134.7, 135.6, 139.9, 142.4, 167.6, 170.3, 171.3, 171.8, 172.2, 174.5, 203.7; FAB HRMS (NBA/Cs) m/e found: 1057.2765 (M + Cs⁺); calc'd for C₅₀H₅₅N₂O₁₅Cs: 1057.2735.

Dansyl taxoid 5a

A solution of compound 4 (4.6 mg, 0.005 mmol) and triethylamine (1 μ l, 0.0075 mmol, 1.5 eq.) in acetonitrile (1 ml) at room temperature under argon was treated with dansyl chloride (2 mg, 0.007 mmol, 1.4 eq.). After 10 min, the solvent was removed *in vacuo* and the residue was purified by flash chromatography (silica, 50 % EtOAc in hexanes) to give 5a as a white powder (4 mg, 69 %): mp = 208–210 °C; R_f = 0.29 (silica, 50 % EtOAc in hexanes); IR (thin film) ν_{\max} 1096, 1382, 1474, 1522, 1744, 1748, 1793, 2958, 3109 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ , 1.12 (s, 3 H, 16-H), 1.19 (s, 3 H, 17-H), 1.73 (s, 3 H, 19-H), 1.78 (s, 3 H, 18-H), 1.91–2.00 (m, 1 H, 6 β -H), 2.15 (s, 3 H, 10-OAc-H), 2.22–2.30 (m, 2 H, 14-H), 2.37 (s, 3 H, 4-OAc-H), 2.50–2.59 (m, 1 H, 6 α -H), 2.86 (s, 6 H, NMe-H), 3.20 (m, 2 H, alanyl CH₂), 3.41–3.49 (m, 2 H, alanyl CH₂), 3.86 (d, J = 7, 1 H, 3-H), 4.14 (d, J = 8, 1 H, 20-H, A of AB), 4.29 (d, J = 8, 1 H, 20-H, B of AB), 4.79 (b m, 1 H, 2'-H), 4.87 (d, J = 9, 1 H, 5-H), 5.50 (dd, J = 7, 10, 1 H, 7-H), 5.64 (d, J = 7, 1 H, 2'-H), 5.77 (dd, J = 2.5, 8, 1 H, 3'-H), 5.87 (br t, J = 6, 1 H, 13-H), 6.17 (s, 1 H, 10-H), 6.18 (br m, 1 H, NH), 7.02 (d, J = 9, 1 H, NH), 7.16 (d, 1 H, J = 7.5, Ar-H), 7.29–7.60 (m, 13 H, Ar-H), 7.74 (d, J = 7.5, 2 H, Bz-H), 8.09 (d, J = 7.5, 2 H, Bz-H), 8.22 (d, J = 7.5, 1 H, Ar-H), 8.31 (d, J = 8.5, 1 H, Ar-H), 8.50 (d, J = 8.5, 1 H, Ar-H); ¹³C NMR (125 MHz, CDCl₃) δ 9.4, 10.7, 14.5, 20.7, 20.8, 20.8, 22.4, 24.8, 25.5, 26.4, 29.6, 33.2, 33.8, 33.9, 35.4, 38.1, 43.0, 45.3, 46.6, 49.0, 54.8, 55.9, 66.4, 71.6, 72.1, 73.0, 74.1, 75.2, 78.4, 80.6, 83.6, 115.0, 119.0, 123.1, 126.9, 128.0, 128.3, 128.6, 128.9, 128.9, 129.0, 129.6, 129.8, 130.0, 131.9, 132.7, 133.5, 133.7, 135.2, 137.8, 140.5, 151.7, 166.8, 169.8, 170.3, 170.8, 172.4, 201.8; FAB HRMS (NBA/Cs) m/e found: 1290.3288 (M + Cs⁺); calc'd for C₆₂H₆₇N₃O₁₇Cs: 1290.3246.

Fluorescein taxoid 5b

A solution of compound 4 (7.4 mg, 0.008 mmol) in 1:1 dioxane:saturated aqueous sodium bicarbonate (1 ml) at room temperature under argon was treated with 6-(fluorescein-5-(and 6)-carboxamidehexanoic acid succinimidyl ester (5 mg, 0.009 mmol, 1.1 eq.). After 1.2 h, the solvent was removed *in vacuo* and the residue was purified by flash chromatography (silica, 1 % AcOH and 10 % MeOH in methylene chloride) to give 5b as a yellow film (7 mg, 65 %): mp = 300 °C (dec.); R_f = 0.41 (silica, 1 % AcOH and 10 % MeOH in methylene chloride); IR (thin film) ν_{\max} 1112, 1451, 1620, 1728, 1734, 2980, 3000, 3401 cm⁻¹; ¹H NMR (500 MHz, 1:1 CDCl₃:CD₃OD) δ , 0.93 (s, 3 H, 16-H), 0.97 (s, 3 H, 17-H), 1.15–1.22 (m, 2 H, CH₂), 1.40–1.50 (m, 4 H, CH₂), 1.59 (s, 3 H, 19-H), 1.60–1.63 (m, 1 H, 6 β -H), 1.79 (s, 3 H, 18-H), 1.97 (s, 3 H, 10-OAc-H), 1.98–2.03 (m, 4 H, CH₂), 2.08–2.16 (m, 2 H, 14-H), 2.17 (s, 3 H, 4-OAc-H), 2.26–2.40 (band, 3 H, 6 α -H, CH₂), 3.16–3.30 (m, 2 H, alanyl

CH₂), 3.69 (d, J = 7, 1 H, 3-H), 4.01 (d, J = 8.5, 1 H, 20-H, A of AB), 4.09 (d, J = 8.5, 1 H, 20-H, B of AB), 4.55 (b m, 1 H, 2'-H), 4.74 (d, J = 9, 1 H, 5-H), 5.38 (dd, J = 7, 10, 1 H, 7-H), 5.48 (d, J = 7, 1 H, 2-H), 5.50 (m, 1 H, 3'-H), 5.96 (br t, J = 9, 1 H, 13-H), 6.03 (s, 1 H, 10-H), 6.28–6.39 (band, 4 H, Ar-H), 6.98 (br t, J = 7, 1 H, NH), 7.02 (d, J = 8, 1 H, Ar-H), 7.10 (m, 1 H, NH), 7.15–7.32 (band, 10 H, Ar-H, NH), 7.57 (d, J = 7.5, 2 H, Ar-H), 7.89 (d, J = 7.5, 2 H, Bz-H), 7.97 (dd, J = 1.5, 7, 2 H, Ar-H), 8.15 (bt, J = 6, 1 H, Ar-H), 8.21 (s, 1 H, Ar-H); ¹³C NMR (125 MHz, 1:1 CDCl₃:CD₃OD) δ 11.0, 14.0, 20.8, 21.0, 22.5, 24.6, 25.9, 26.0, 28.2, 33.1, 33.2, 33.9, 34.0, 35.1, 35.7, 35.8, 39.1, 39.3, 39.5, 39.9, 43.3, 47.2, 50.8, 51.0, 55.1, 56.0, 72.2, 72.3, 72.8, 74.0, 75.3, 76.1, 79.8, 80.7, 83.2, 102.5, 126.4, 127.2, 128.0, 128.1, 128.2, 128.3, 128.3, 128.4, 129.4, 131.8, 132.4, 133.0, 140.7, 141.2, 166.4, 166.6, 170.0, 171.0, 178.8, 199.1, 202.4; FAB HRMS (NBA/Cs) m/e found: 1528.4010 (M + Cs⁺); calc'd for C₇₇H₇₇N₃O₂₂Cs: 1528.4053.

Carboxyrhodamine taxoid 5c

A solution of compound 4 (7.4 mg, 0.008 mmol) in 1:1 dioxane:saturated aqueous sodium bicarbonate (1 ml) at room temperature under argon was treated with (6-(and 6)-carboxysuccinimidyl ester)-2-carboxyrhodamine (6 mg, 0.009 mmol, 1.1 eq.). After 1.5 h, the solvent was removed *in vacuo* and the residue was purified by HPLC (a gradient of 80 % 20 mM ammonium acetate in methanol to 100 % methanol over 30 min) to give 5b as a red film (8 mg, 73 %): mp = 252–256 °C (dec.); R_f = 0.58 (silica, 1 % AcOH and 10 % MeOH in methylene chloride); IR (thin film) ν_{\max} 1064, 1156, 1550, 1601, 1720, 1756, 2986, 3690 cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ , 0.85–0.95 (m, 4 H, CH₂), 1.06 (s, 3 H, 16-H), 1.14 (s, 3 H, 17-H), 1.20–1.41 (m, 4 H, CH₂), 1.76 (s, 3 H, 19-H), 1.89 (s, 3 H, 18-H), 2.05 (m, 1 H, 6 β -H), 2.06 (m, 2 H, CH₂), 2.09 (s, 3 H, 10-OAc-H), 2.35 (s, 3 H, 4-OAc-H), 2.51–2.70 (band, 3 H, CH₂, 6 α -H), 3.01–3.04 (m, 2 H, CH₂), 3.41–3.71 (m, 6 H, CH₂), 3.91 (d, J = 7, 1 H, 3-H), 4.16 (d, J = 8.5, 1 H, 20-H, A of AB), 4.28 (d, J = 8.5, 1 H, 20-H, B of AB), 4.48 (b m, 1 H, 2'-H), 4.74 (d, J = 9, 1 H, 5-H), 5.60 (band, 3 H, 2-H, 3'-H, 7-H), 6.12 (br t, J = 9, 1 H, 13-H), 6.22 (s, 1 H, 10-H), 6.73 (d, J = 7.5, 1 H, Ar-H), 7.25 (br t, J = 9, 1 H, NH), 7.40–7.72 (band, 13 H, Ar-H), 7.81 (d, J = 8, 1 H, Ar-H), 8.00 (d, J = 9, 1 H, Ar-H), 8.12 (d, J = 7.5, 2 H, Ar-H), 8.44 (s, 1 H, Ar-H); ¹³C NMR (125 MHz, CD₃OD) δ 11.0, 13.1, 14.7, 20–21 (m), 22.0, 25.1, 25.3, 28–29 (m), 30.2, 31.3, 43.0, 50.1, 51.5, 55.1, 56.0, 65.1, 100.2, 104.7, 110.6, 111.3, 114.0, 119.3, 122.4, 124.0, 125.7, 126.0, 126.4, 127.0, 127.1, 127.3, 128.2, 129.0, 130.3, 131.4, 133.2, 133.4, 134.7, 151.2, 152.0, 158.0, 166.3, 168.0, 169.2, 171.1, 173.4, 206.2; positive electrospray m/e found: 1442.6 (M + H⁺); calc'd for C₈₃H₈₆N₄O₁₉: 1442.0.

Sulforhodamine (Lissamine Red) taxoid 5d

A solution of compound 4 (19 mg, 0.021 mmol) in 1:1 dioxane:saturated aqueous sodium bicarbonate (1.5 ml) at room temperature under argon was treated with 6-chlorosulfonyl-2-sulfoxytetraethyl-rhodamine (Lissamine Rhodamine B chloride) (15 mg, 0.026 mmol, 1.25 eq.). After 2 h, the mixture was diluted with aqueous sodium chloride (5 ml) and extracted (10 \times 10 ml) with ethyl acetate. The combined organic layer was evaporated and the residue purified by HPLC (a gradient of 80 % 20 mM ammonium acetate in methanol to 100 % methanol over 30 min) to give compound 5b as a deep purple film (22 mg, 75 %): mp > 300 °C; R_f = 0.54 (silica, 1 % AcOH and 10 % MeOH in methylene chloride); IR (thin film) ν_{\max} 1064, 1156, 1550, 1601, 1720, 1756, 2986, 3690 cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ , 0.97 (s, 3 H, 16-H), 1.01 (s, 3 H, 17-H), 1.16 (m, 12 H, CH₃CH₂N), 1.61 (s, 3 H, 19-H), 1.61–1.64 (m, 1 H, 6 β -H), 1.74 (s, 3 H, CH₃CH₂N), 1.81–1.85 (m, 1 H, 14-H), 2.04 (s, 3 H, 10-OAc-H), 2.03–2.12 (m, 1 H, 14-H), 2.21 (s, 3 H, 4-OAc-H), 2.33–2.47 (band, 3 H, CH₂, 6 α -H), 3.10–3.18 (m, 2 H, CH₂), 3.53 (br t, 8 H, J = 7, CH₂N), 3.76 (d, J = 7, 1 H, 3-H), 4.06 (dd, J = 8.5, 12, 2 H, 20-H, AB), 4.60 (d, J = 5, 1 H, 2'-H), 4.85 (d, J = 9, 1 H, 5-H), 5.42–5.5 (band, 3 H, 2-H, 3'-H, 7-H), 6.01 (br t, J = 9, 1 H, 13-H), 6.09 (s, 1 H, 10-H), 6.80 (brs, 2 H, Ar-H), 6.84 (m, 2 H, Ar-H), 6.97 (m, 2 H, Ar-H), 7.17 (br t, J = 9, 1 H, NH), 7.22–7.43 (band, 12 H, Ar-H), 7.53 (m, 1 H, Ar-H), 7.72 (d, J = 8, 1 H, Ar-H), 8.01 (d, J = 9, 1 H, Ar-H), 8.03 (dd, J = 2, 7, 1 H, Ar-H), 8.54 (br s, 1 H, Ar-H); ¹³C NMR

(125 MHz, CD₃OD) δ 11.6, 12.9, 14.8, 21.0, 22.8, 23.6, 26.7, 34.2, 35.4, 36.2, 39.0, 44.6, 46.8, 47.2, 56.9, 57.8, 72.3, 73.4, 74.9, 76.0, 77.1, 77.4, 78.5, 82.1, 85.3, 97.0, 115.0, 115.1, 116.0, 116.1, 127.5, 128.5, 129.0, 129.4, 129.6, 129.8, 131.2, 131.3, 132.0, 132.9, 133.1, 133.2, 133.9, 134.4, 135.5, 135.6, 140.0, 142.5, 144.0, 147.3, 157.2, 157.3, 158.0, 159.6, 167.6, 170.6, 171.0, 171.3, 172.1, 174.3, 204.0; positive electrospray *m/e* found: 1487.0 (*M* + *H*⁺); calc'd for C₇₇H₈₅N₄S₂O₂₁: 1487.0.

Solubility measurements

The taxoid (1 mg) was placed in a 2-ml Eppendorf tube and suspended in deionized water (1 ml) with the aid of sonication for 5 min. The tubes were spun at 14 000 rpm for 1 min in a microcentrifuge to settle particulate matter. The solution was then carefully removed and filtered through a syringe filter (Dupont, 0.45 μ m PTFE). Solubility was determined by measuring the UV absorbance of an aliquot of the aqueous solution at 254 nm and comparing to a standard curve produced by standard dilution of a 1 mM solution of the taxoid in methanol.

Partition coefficient

After the solubility of the taxoid was measured as above, the solution was replaced in the original Eppendorf tube, nonanol (1 ml) was added, and the mixture shaken vigorously for 5 min. The tubes were centrifuged to settle any particulate matter and the layers were separated. Partition coefficients were determined by comparing the UV absorbance of the layers at 254 nm.

Stability

The stability of the taxoids was measured by thin layer chromatography of a 1 mM solution in ethanol. The solutions were kept at ambient temperature under air and the purity checked each day. The experiment was discontinued after two weeks when no significant degradation was detected. The stability was confirmed by examining the ¹H NMR spectra of each compound at the end of the experiment.

Biology

Cytotoxicity measurements

Cells (ATTC HeLa and CHO, 1250 per well) were plated on 96-well plates in RPMI media with fetal calf serum (FCS) (245 μ l per well) and grown to ~50 % confluence (24 h). The taxoids were diluted in ethanol and added as 5 μ l aliquots directly to the wells to give final concentrations ranging from 1×10^{-4} to 1×10^{-10} M for each compound. The experimental controls were ethanol (5 μ l) as the negative cytotoxicity control and sodium dodecylsulfate (1×10^{-3} M final concentration) as the positive cytotoxicity control. The growth inhibition was determined using the standard XTT assay [41].

Tubulin polymerization assays

Microtubules were prepared from the brains of 1–3 day old baby chicks (Hall Bros. Hatchery, Norwich, CT) by the standard double polymerization–depolymerization cycling in PM buffer [39,47]. On the day of a polymerization experiment, the microtubules and overlay buffer were thawed quickly. As the buffer thawed, the pellet was resuspended in the overlay buffer by gentle trituration with a Pasteur pipet. A final round of assembly at 37 °C followed by disassembly and clarification at 4 °C was performed to select for assembly competent protein after the period of storage. The resulting solution of 3 \times assembled microtubules (tubulin plus MAPs) was used in the assembly experiments reported herein. Polymerization assays were carried out on three-cycled tubulin in PIPES buffer (100 mM, pH 6.9) containing GTP (2 mM), MgSO₄ (5 mM), and EGTA (1 mM) [41]. The protein was not further purified and thus retained the MAPs. The tubulin solution was treated with taxoid (10 μ M) and allowed to polymerize at 37 °C while the optical density was monitored at 340 nm.

Microscopy

Human foreskin fibroblasts were treated at 37 °C with taxoids at 20 μ M, a concentration that is equimolar with that of cellular tubulin (E.D. Salmon, personal communication). After 1 h or 24 h, cells were observed

with a Zeiss Axiophot microscope using differential interference contrast and fluorescence optics via a 40 \times 1.3 N.A. objective or a 100 \times 1.4 N.A. objective. Color micrographs were recorded on Kodak Ektachrome exposed at an ASA of 400 and developed commercially. Confocal microscope images were generated by a BioRad MRC 1000 operating on a Nikon microscope with a 63 \times 1.4 N.A. objective and captured electronically and stored on magnetic media. Composite color figures were produced either from stored electronic images or by scanning color photographs and manipulating the resulting files with Adobe Photoshop. Cells were fixed first in 3.7 % formaldehyde in PBS (phosphate buffered saline, pH 7.0) for 15 min at room temperature and then in 100 % methanol at –20 for 10 min. Following the methanol fixation and permeabilization step, the cells were rehydrated in PBS and then stained with a monoclonal antibody to α tubulin (Sigma Chemical Co.) followed by the appropriate fluorescent secondary antibody (Kirkegaard and Perry, Inc.). Both the primary and secondary antibodies were used at the dilutions recommended by the respective suppliers of the antibodies. For images of living cells, the fixation and permeabilization steps were omitted.

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